INFLUENCE OF HERBICIDES ON LIPID RESERVES, MORTALITY AND INFECTIVITY OF *HETERORHABDITIS AMAZONENSIS* (RHABDITIDA: HETERORHABDITIDAE)

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Summary. Infective juveniles (IJs) of entomopathogenic nematodes show reduced infectivity when their lipid content decreases. The expenditure of this energy reserve can be accelerated by stress factors, such as contact with chemical pesticides. The aim of this study was to correlate the amount of lipids in *Heterorhabditis amazonensis* IJs with their mortality and infectivity after exposure to solutions of the herbicides clomazon + hexazinon (Ranger), at the rate of 0,0027 g, and of simazine + ametryn (Topeze) at the rate of 0,0054 ml, in 200 ml sterilized distilled water (equivalent to 4 kg/ha of Ranger and 8 l/ha of Topeze). Nematodes in distilled water were used as a control. Nematode mortality was evaluated by observing 1,000 IJs randomly. Infectivity was evaluated by inoculating 1 ml of nematode suspension on *Galleria mellonella* larvae. Lipids in IJs were stained with "Oil Red O" dye, mounted on slides, and photographed with a trinocular microscope. The area corresponding to the lipid reserve was measured with the "Image Tool" software. The results showed that the herbicides did not kill *H. amazonensis* IJs after 5 days exposure to them, nevertheless they reduced IJ infectivity. The amount of lipids in IJs exposed to the herbicides was smaller than in IJs kept in water and appeared to be correlated with reduced infectivity.

Keywords: Ametryn, clomazon, biological control, entomopathogenic nematodes, hexazinon, simazine.

Entomopathogenic nematodes (EPNs) (Rhabditida) are important because of their association with symbiont bacteria, which are introduced by the infective juveniles (IJs) into the insect's hemocoel and cause septicemia and host death in 24 to 48 hours (Adams and Nguyen, 2002; Ferraz, 1998). Therefore, they can be used in integrated pest management programmes as biological control agents. As such they are applied in areas that receive different agricultural inputs, such as chemical products, fertilizers, and soil pH-correcting materials.

Infective juveniles of EPNs are known to be tolerant to short exposure to herbicides, insecticides, fungicides, and miticides. However, there are differences of susceptibility among EPNs depending on the species (Grewal *et al*., 1998). The chemical substances employed to control pests and diseases can reduce the control efficacy of EPNs (Alves *et al*., 1998; Grewal *et al*., 2001) or be synergistic, since at sub-lethal doses they can cause stress on the insect pest and provide better control by the nematode (Hara and Kaya, 1983; Kaya *et al*., 1995; Koppennhöfer and Kaya, 1998).

Lipids are energy reserves in nematodes that live in aerobic habitats, and are the main source of energy in IJs, with total content values ranging from 11 to 67% of their dry weight (Barrett and Wright, 1998; Chitwood, 1998). Differences in lipid composition can lead to differences in the physiology of EPNs, such as survival and pathogenicity (Hatab and Gaugler, 1999; Menti *et al*., 2003). Using these reserves, nematodes can remain alive until they find a new host to parasitize (Lee and Atkinson, 1977; Van Gundy, 1985).

However, as lipid reserves are depleted the infectivity of IJs also decreases (Fitters and Griffin, 2004). The expenditure of this energy reserve can be accelerated due to stress factors, such as contact with chemical products (Patel *et al*., 1997; Andaló, 2006).

The selection of EPNs for commercial use in biological control is based on knowledge about their physiological and biochemical attributes (Wright and Perry, 2002). The herbicides clomazon + hexazinon (Ranger) and simazine + ametryn (Topeze) are widely used in the Brazilian agricultural sector, both being recommended for use on sugarcane (*Saccharum* spp.) and Topeze also for use on coffee (Andrei, 2005). On organic farms, the use of fungi, predators and parasitoids is recommended for the control of insect pests of these crops. In sugarcane, EPNs are being tested to control *Migdolus fryanus* Westwood (Coleoptera: Cerambycidae) and in coffee to control *Dysmicocccus texensis* (Tinsley, 1900) (Hemiptera: Pseudococcidae) (Andaló *et al*., 2004b; Machado *et al*., 2005). Because pesticides can affect the efficacy of EPNs, as noted above, assessment of the effects of herbicides on EPNs is extremely relevant to planning the use of EPNs for pest control.

Therefore, the objective of this study was to correlate the amount of lipid reserves in the EPN *Heterorhabditis amazonensis* Andaló, Nguyen *et* Moino Jr, 2006 with its survival and infectivity when exposed to herbicides. This nematode is being exploited for its potential in the control of several insect pests of crops, including those mentioned above.

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MATERIALS AND METHODS

Multiplication of EPNs

The suspension in which the nematodes used in the experiment were raised was kept in Erlenmeyer flasks in a BOD incubator at 16 °C, in an aqueous suspension containing approximately 500 IJs/ml.

Heterorhabditis amazonensis RSC5 was obtained from the entomopathogenic microorganisms bank, Insect Pathology Laboratory, Departamento de Entomologia (UFLA) and multiplied on *Galleria mellonella* L. (Lepidoptera: Pyralidae) caterpillars reared at the afore mentioned laboratory, according to the methodology described by Dutky *et al*. (1964), using the artificial diet modified by Parra (1998).

Ten of these caterpillars, approximately 2 cm long, were selected and placed in a Petri dish (9 cm diameter), with two sheets of filter paper in the bottom; 1 ml of nematode suspension was inoculated onto the paper to give 20 ± 5 IJs/caterpillar. The dishes were maintained in a BOD incubator for 72 h, at 24 ± 1 °C and a 24 h in the dark until the caterpillars died. The caterpillars were then removed and placed for 4 days at 24 °C in 9-cm diameter Petri dishes containing dry filter paper (Molina and López, 2001).

Later, ten caterpillars killed by nematodes were placed in a modified White trap (White, 1927), with about 3 ml water on the bottom of the dish. The traps were placed in a BOD incubator at 24 ± 1 °C for 3 to 5 days. The suspension collected daily was transferred to a 1000 ml graduated cylinders containing 800 ml distilled water. The IJs settled on the bottom of the graduated cylinder after 24 h were then collected. Next, the nematode suspension was quantified in polystyrene ELISA plates.

Assessment of the effects of the herbicides on the EPNs

Different methods can be used to determine neutral lipid contents, including chemically, by chromatography, or histologically, using "Oil Red O" dye (Lee, 1960; Croll, 1972; Christophers *et al*., 1997). Chemical methods have the disadvantage of being very time-consuming and require large quantities of nematodes. Staining nematodes with a dye specific to lipid (Oil Red O) generates a red area corresponding to the presence of lipids. The amount of the lipid reserve in the stained area can be determined with image analysis (Stamps and Linit, 1995). This method was used in our experiment.

The method adapted from the protocol developed by Vainio (1992) was used to evaluate the effects of the herbicides on survival and infectivity of EPNs in the laboratory.

Two herbicides were used. They were clomazon + hexazinon (as Ranger WG, clomazon 40% and hexazinon 10%) and simazine + ametryn (as Topeze SC, simazine 19,5% and ametryn 19,5%). Nematodes maintained in sterilized distilled water were used as a control. Treatments were arranged according to a completely randomized design and each consisted of five replicates.

Solutions of each herbicide were prepared at double the largest recommended concentration of the active ingredient (Ranger: 4 kg/ha; Topeze: 8 l/ha), equivalent to 0,0027 g of Ranger or 0,0054 ml of Topeze in 200 ml sterilized distilled water. Then 1 ml of the solution was placed in a glass vial (30 ml volume) and 1 ml of the nematode suspension in distilled water, containing about 2,500 IJs, was added. The vials were maintained in a BOD incubator at 24 ± 1 °C.

Effect on nematode mortality. Five days after incubation, 1 ml of the suspension was taken, 1,000 randomly selected IJs were observed, and the numbers of dead and live individuals were counted. Nematodes showing no movement and those that did not move after adding 0.1 ml NaOH 1N were considered dead (Chen and Dickson, 2000). Nematode mortality percentages were then calculated.

Effect on EPN infectivity. To assess nematode infectivity, 3 ml of sterilized distilled water were added to another set of vials (five replicates per treatment), each containing 2 ml of nematodes and herbicide suspension, and the mixture was left to settle for half an hour in a refrigerator at 8 °C. About 3 ml of the supernatant were discarded. This procedure was repeated three times to eliminate herbicide residues. Then, 1 ml was taken from the bottom of each vial and pipetted onto a filter paper in a 5-cm-diameter Petri dish containing five *G. mellonella* caterpillars. The same procedure was performed in the control treatment.

The dishes were maintained in a BOD incubator at 24 ± 1 °C for four days. The dead caterpillars were removed and placed in Petri dishes lined with dry filter paper, maintained in the dark for 2 days, and then dissected to ascertain the presence of the nematodes to be sure that death was caused by nematodes.

Effect on lipid content of EPNs. Lipids were quantified by taking 1 ml of the nematode suspension from the same vials used in the infectivity test. The IJ body concentration of lipids was determined by the colorimetric method, using Oil Red O dye (Christophers *et al*., 1997; Storey, 1983). For this, a solution containing 0.5 g of the dye in 100 ml of absolute alcohol was prepared by agitation for 15 minutes and then filtering through a Whatman no. 1 filter paper. The solution was stored at 5 °C in a dark glass flask to protect it from light. The IJ suspension was concentrated by settling to 0.5 ml and 3 ml of the Oil Red O dye solution were added to it. The mixture was next heated in a water bath at 60 °C for 20 min and then was cooled at room temperature and the nematodes were allowed to settle. Next, about 2.5 ml of the supernatant were discarded, and 3 ml of a distilled water + pure glycerin solution (1:1) were added to the nematodes left in the bottom of the vial, and the suspension was then stored at room temperature.

One slide was prepared for each replicate by placing five nematodes on the slide and three nematodes selected at random were used to assess the effect of the herbicides on lipid reserves of the nematodes. The full area of the nematode's body and the red-stained area, corresponding to lipids, were obtained by running the photographs through the "Image Tools for Windows" software, version 3.0. Measurement of the area of red staining allowed us to infer lipid percentage in relation to the full IJ body area.

Statistical analysis

The mortality, infectivity, and lipid percentage data thus obtained were submitted to analysis of variance and the Scott and Knott test (1974) at 5% probability for comparisons between means.

RESULTS AND DISCUSSION

The herbicides tested (Topeze and Ranger), at the concentrations studied, did not cause ($P<0.05$) mortality of *H. amazonensis* IJs after exposure of the nematodes to them for five days (Fig. 1). However, the IJs incubated in the herbicide solutions had their infectivity reduced ($P<0.05$) as compared to the IJs stored in water for the same period (Fig. 2). In addition, the lipid content was less ($P<0.05$) when the IJs were incubated in the herbicides as compared with those stored in water (Fig. 3). Andaló *et al.* (2004a) also incubated *H. bacteriophora* Poinar IJs in the same herbicides for 7 days without causing mortality.

The IJs exposed for 5 days to Ranger and Topeze caused 52 and 56% infectivity on *G. mellonella*, respectively, while non-treated (control) nematodes showed infectivity of 100% (Fig. 2). There was smaller lipid content in IJs treated with Ranger (52%) and Topeze (48%) as compared with the control (86%). Therefore, besides reducing infectivity, the herbicides caused losses of energy reserve without causing mortality. Lipids are the main source of energy in IJs. Using these reserves nematodes will remain alive until they can find a new host to parasitize. The level of lipid reserves maintained interferes directly with nematode infectivity (Lee and Atkinson, 1977; Van Gundy, 1985).

The decreased infectivity of *H. amazonensis* indicates that the IJs were affected by exposure to the herbicides. A reduction in nematode infectivity on *G. mellonella* larvae was also observed by Head *et al.* (2000) for the nematode *Steinernema carpocapsae* (Weiser) Wouts, Mráček, Gerdin *et* Bedding. This nematode was exposed to the insecticides abamectin, deltamethrin, dimethoate, heptenophos, and trichlorfon, and tested under laboratory conditions against *G. mellonella*. The assays showed a reduction in nematode infectivity when it was exposed to the chemical products, with a high caterpillar survival index.

The reduction in lipid content of IJs stored in solu-

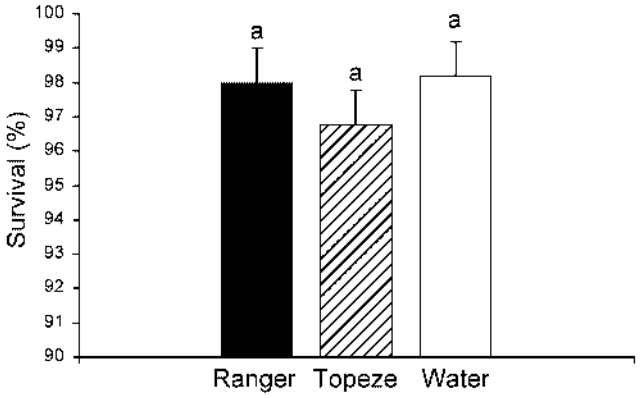

Fig. 1. Survival (%) of *Heterorhabditis amazonensis* maintained for 5 days in water or exposed to the herbicides Ranger or Topeze. Bars followed by different letters are different by the Scott and Knott test ($P<0.05$).

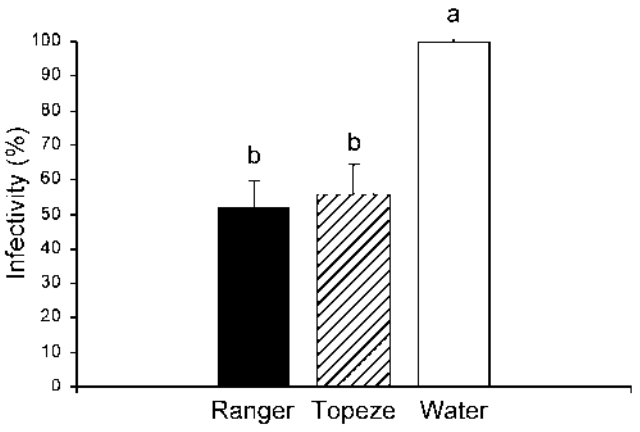

Fig. 2. Infectivity (%) of *H. amazonensis* in *Galleria mellonella* larvae, after exposure for 5 days to the herbicides Ranger or Topeze as compared with water (control). Bars followed by different letters are different by the Scott and Knott test ($P<0.05$).

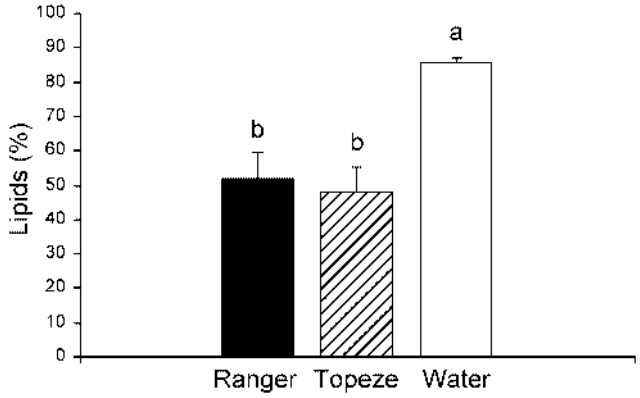

Fig. 3. Neutral lipid content (%) in *H. amazonensis* infective juveniles after storage for 5 days in solutions of the herbicides Ranger or Topeze, as compared with water (control). Bars followed by different letters are different by the Scott and Knott test ($P<0.05$).

tions of the tested herbicides demonstrates a relationship between lipid content and infectivity of the nematode but without any effect on IJ mortality.

Hara and Kaya (1983) evaluated the effect of the chemicals mevinphos, fenamiphos, trichlorfon, oxamyl, and methomyl on *S. carpocapsae* infectivity on larvae of *Spodoptera exigua* Hubner (Lepidoptera: Noctuidae). They observed a decrease in nematode infectivity to the larvae of the insect. Andaló (2006) observed a reduction of the infectivity of *Heterorhabditis* spp. to *G. mellonella* larvae as lipid content of the nematode decreased. Hass *et al*. (2002) also observed that the reduction of infectivity of IJs of the genus *Heterorhabditis* is associated with a loss of lipid reserves in these nematodes.

All the above clearly demonstrates that the quality and quantity of IJ lipids are particularly important for the use of EPNs as pest control agents, since they critically influence nematode viability and infectivity (Wright and Perry, 2002). The incompatibility of the products Ranger and Topeze with the nematodes tested might be related to their mode of action, since they are reported to act by inhibiting chlorophyll synthesis through the lysis of cell membranes. Such disorganization and membrane lyses are mainly caused by lipid breakdown; therefore, the products would act directly as lipid synthesis inhibitors (Moorman, 1994; Scalla and Matringe, 1994).

The possibility of using EPNs in integrated pest management programmes (IPM) depends on careful study of the selection of adapted species or populations to provide information on their physiological and biochemical attributes, and so to allow their efficiency to be increased for commercial use. Consequently, the use of EPNs in association with chemical products should take into consideration their compatibility, since there could be a reduction in control efficiency due to decreased nematode infectivity.

Our results indicated that the use of the herbicides Ranger and Topeze and the EPN *H. amazonensis* at the same time may not be wise. The application of the nematodes long before or after that of the herbicides could avoid any problems, but this approach needs to be shown to be effective under field conditions.

ACKNOWLEDGMENTS

To Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for awarding a postdoctoral fellowship to the first author.

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Accepted for publication on 19 January 2009.